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(71) Applicant (for all designated States except US): PRESIDENT
AND FELLOWS OF HARVARD COLLEGE [US/US]; 17
Quincy Street, Cambridge, MA 02138 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): WILEY, Don, C.
[US/US]; 16 Brown Street, Cambridge, MA 02138 (US).
BOUVIER, Marlene [US/US]; 2 Ware Street, #508,
Cambridge, MA 02138 (US).

(74) Agent: TSAO, Y., Rocky; Fish & Richardson P.C., 225
Franklin Street, Boston, MA 02110-2804 (US).

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(57) Abstract

A synthetic MHC-blocking peptide identical to an antigenic peptide of the T-cell receptor except that (i) a linker is covalently bonded to a first amino acid residue and a second amino acid residue of the peptide to form a ring, and that (ii) each of the first and second amino acid residues contains a side chain with a functional group via which the first and second amino acid residues are covalently bonded to the linker; or except that (i) one, two, or three chains are respectively linked via covalent bonding to one two, or three amino acid residues of the peptide, and that (ii) each of the one, two, or three amino acid residues has a side chain with a functional group via which each of the one, two, or three chains is covalently linked.

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NONIMMUNOGENIC MHC-BLOCKING PEPTIDES**CROSS REFERENCE TO RELATED APPLICATIONS**

Under 35 USC § 120, this application claims the
5 benefit of prior U.S. provisional application 60/000,266
filed June 16, 1995

BACKGROUND OF THE INVENTION

Antigenic peptides of T cells first bind to class
I or class II major histocompatibility complex (MHC)
10 molecules before interacting with T cell receptors on CD⁸⁺
T cells or CD⁴⁺ T cells to elicit immune responses.

Stern, L.J. & Don C. Wiley (1994), Structure 2, 245-251.

In general, conservative residue substitutions in
the middle portion of antigenic peptides of T cells are
15 introduced to selectively inhibit activation of T cells
without significantly altering binding affinity to the
relevant MHC molecules. See Sloan-Lancaster, J.,
Evavold, B.D. & Allen, P.M. (1993) Nature 363, 156-159;
De Magistris, M.T., Alexander, J., Coggeshall, M.,
20 Altman, A., Gaeta, F.C.A., Grey, M.H. & Sette, A. (1992)
Cell 68, 625-634; and Alexander, J., Snoke, K., Ruppert,
J., Sidney, J., Wall, M., Southwood, S., Oseroff, C.,
Arrhenius, T., Gaeta, F.C.A., Colon, S.M., Grey, H.M. &
Sette, A. (1993) J. Immunol. 150, 1-7.

25 Alternatively, other designs of MHC-blocking
peptides have included the replacement of several
residues from the middle portion of antigenic peptides
with moieties such as 4-aminobutyric acid,
6-aminohexanoic acid, and phenantridine to act as
30 spacers. E.g., see Rognan, D., Scapozza, L., Folkers, G.
& Daser, A. (1995) Proc. Natl. Acad. Sci. USA 92,
753-757.

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SUMMARY OF THE INVENTION

The present invention features MHC-blocking peptides which are designed based on novel modifications of antigenic peptides capable of binding to class I or
5 class II MHC molecules for presentation to CD⁸⁺ or CD⁴⁺ T cells.

An aspect of this invention therefore relates to a synthetic peptide that blocks the interaction of a T cell receptor on CD⁸⁺ T cells with an MHC molecule, the peptide
10 containing 8, 9 or 10 amino acid residues and being identical to an antigenic peptide of the T cell receptor except that (i) a linker is covalently bonded to a first amino acid residue and a second amino acid residue of the peptide to form a 20- to 200-membered ring (preferably, a
15 40- to 120-membered ring), and that (ii) each of the first and second amino acid residues contains a side chain with a functional group selected independently from the group consisting of an amino group, a carboxyl group, a hydroxyl group, and a sulfhydryl group. The first and
20 second amino acid residues are covalently bonded to the linker via the just-mentioned functional groups. Furthermore, if the peptide contains 8 amino acid residues, each of the first and second amino acid residues is at one of positions 3-8 (e.g., 3-7 or 4-8);
25 if the peptide contains 9 amino acid residues, each of the first and second amino acid residues is at one of positions 4-8; and if the peptide contains 10 amino acid residues, each of the first and second amino acid residues is at one of positions 4-9 (e.g., 4-8 or 5-9).
30 Preferably, the first and second amino acid residues are apart by 2 or 3 amino acid residues.

Another aspect of this invention relates to a synthetic peptide that blocks the interaction of a T cell receptor on CD⁸⁺ T cells with an MHC molecule, the peptide
35 containing 8, 9 or 10 amino acid residues and being

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identical to an antigenic peptide of the T cell receptor except that (i) one, two, or three chains (preferably, two or three chains), each 10-200 atoms (preferably, a 20-100 atoms) in length, are respectively linked via
5 covalent bonding to one, two, or three amino acid residues (preferably, two or three amino acid residues) of the peptide, and that (ii) each of the one, two, or three amino acid residues has a side chain with a functional group selected independently from an amino
10 group, a carboxyl group, a hydroxyl group, and a sulfhydryl group. Each of the one, two, or three chains is covalently linked to the just-mentioned functional group of each of the one, two, or three amino acid residues. Furthermore, if the peptide contains 8 amino
15 acid residues, each of the one, two, or three amino acid residues is at one of positions 3-8 (e.g., 3-7 or 4-8); if the peptide contains 9 amino acid residues, each of the one, two, or three amino acid residues is at one of positions 4-8; and if the peptide contains 10 amino acid
20 residues, each of the one, two, or three amino acid residues is at one of positions 4-9 (e.g., 4-8 or 5-9). When two or three chains are attached to the peptide backbone, it is preferred that the anchor positions be apart by 2 or 3 amino acid residues.

25 A further aspect of this invention relates to a synthetic peptide that blocks the interaction of a T cell receptor on CD⁴⁺ T cells with an MHC molecule, the peptide containing 11-18 amino acid residues and being identical to an antigenic peptide of the T cell receptor except
30 that (i) a linker is covalently bonded to a first amino acid residue and a second amino acid residue of the peptide to form a 20- to 200-membered ring (preferably, a 40- to 120-membered ring), and that (ii) each of the first and second amino acid residues contains a side
35 chain with a functional group selected independently from

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the group consisting of an amino group, a carboxyl group, a hydroxyl group, and a sulfhydryl group. The first and second amino acid residues are covalently bonded to the linker via the just-mentioned functional groups and are
5 located at the middle portion of the peptide backbone (i.e., they are not among the three amino acid residues at either end). Preferably, the first and second amino acid residues are apart by two or more amino acid residues.

10 Also within the scope of this invention is a synthetic peptide that blocks the interaction of a T cell receptor on CD⁴⁺ T cells with an MHC molecule, the peptide containing 11-18 amino acid residues and being identical to an antigenic peptide of the T cell receptor except
15 that (i) one to five chains (preferably, two to four chains), each 10-200 atoms (preferably, a 20-100 atoms) in length, are respectively linked via covalent bonding to one to five amino acid residues (preferably, two to four amino acid residues) of the peptide, and that (ii)
20 each of the one to five amino acid residues has a side chain with a functional group selected independently from an amino group, a carboxyl group, a hydroxyl group, and a sulfhydryl group. Each of the one to five chains is covalently linked to the just-mentioned functional group
25 of each of the one to five amino acid residues, which are located at the middle portion of the peptide backbone (i.e., they are not among the three amino acid residues at either end). When two or more chains are attached to the peptide backbone, it is preferred that the anchor
30 positions be apart by two or more amino acid residues.

What is meant by "synthetic peptide that blocks the interaction of a T cell receptor on T cells with an MHC molecule" (i.e., "synthetic MHC-blocking peptide") is a synthetic peptide which binds specifically to a class I
35 r class II MHC molecule in a manner similar to an

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antigenic peptide of that T cell receptor, but does not activate the T cells. Note that "an antigenic peptide of a T cell" mentioned herein can be either a naturally occurring peptide or a variant thereof (e.g., containing an acetylated α -amino group, an amidated α -carboxyl group, a D- and/or β - or γ -amino acid residue, or any suitable organic moiety); indeed, there are only two criteria, i.e., it is a peptide and it binds to a class I or class II MHC molecule in the manner described in Stern, L.J. & Don C. Wiley, Structure (1994) 2, 245-251.

The anchor amino acid residue to which a linker or a chain is attached via the functional group on its side chain can be either of a naturally occurring amino acid or a synthetic amino acid (e.g., a D-amino acid, a γ -amino acid, or any suitable organic moiety). Examples include, but are not limited to, lysine (Lys), ornithine (Orn), glutamic acid (Glu), aspartic acid (Asp), serine (Ser), threonine (Thr), tyrosine (Tyr), and cysteine (Cys). When three-letter or one-letter symbols are used in this disclosure, L-amino acids are intended unless indicated otherwise. Also note that it is possible that an anchor amino acid residue is the same as its counterpart in the antigenic peptide. For example, when an antigenic peptide has a Lys residue at the anchor position, then there is no need to replace that Lys residue with another anchor amino acid residue. In this disclosure, the position of an anchor amino acid residue is numbered in such a manner that the N-terminal residue is designated as position 1, its neighboring amino acid position 2, and so on. When two anchoring amino acids are apart by two or three amino acid residues, there are two or three amino acid residues between them. Thus, two anchoring amino acids at positions 4 and 8 of a nonapeptide are apart by three amino acid residues (i.e., at positions 5, 6, and 7).

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Examples of a linker include, but are not limited to, a terminally functionalized poly(ethylene glycol) (PEG) such as PEG dicarboxylic acid, poly(D- or L-amino acid), polysaccharide, or polynucleotide, which are
5 reactive at both ends. On the other hand, similar polymer derivatives which are reactive only at one end (e.g., monomethoxy PEG, or mPEG) can be used as chains. The length of a chain refers to the number of atoms that make up its backbone.

10 Other features or advantages of the present invention will be apparent from the following drawings and detailed description of representative examples.

BRIEF DESCRIPTION OF THE DRAWINGS

The drawings are first described.

15 Fig. 1 is a FAB mass spectrum for purified Tax 300 peptide (to be described below) showing a series of peaks for $[M + H]^+$ ions. Each peak is identified by a value of n representing the number of repeating monomer unit in PEG loops.

20 Fig. 2 is gel filtration chromatograms for HLA-A2 complexed with Tax 300, Tax 400, and Tax 600 peptides (all to be described below). Each complex eluted as a single peak is identified by an arrow.

Fig. 3 is a FAB mass spectrum for purified HLA-A2
25 complexed with Tax 300 peptide showing a series of peaks for $[M + H]^+$ peptide ions (compare with spectrum shown in Fig. 1).

DETAILED DESCRIPTION OF THE INVENTION

Activation of T cell receptors on $CD8^+$ and $CD4^+$ T
30 cells requires the recognition of antigenic peptides bound in the groove of MHC molecules on the surface of

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antigen-presenting cells. Antigenic peptides bind in an extended conformation to class I and class II MHC molecules. X-ray crystallography studies have revealed that for all class I MHC complexes, the peptide N- and C-termini form hydrogen bonds with conserved MHC amino acid residues located at both ends of the binding site, some peptide side chains are buried in deep pockets along the binding site, and the central region of the peptide arch away from the floor of the binding site with side chains generally pointing toward T cell receptors. Antigenic peptides of 8- to 10-amino acids long have been shown to adopt this mode of binding. A decamer has been shown to extend out of the binding groove at the C-terminus and an octamer has been shown to preferentially leave the N-terminal end of the binding site unoccupied. On the basis of these observations, other modes of binding can be described: extension of a decamer out of the N-terminal end of the binding site and binding of an octamer preferentially at the N-terminal end of the groove leaving the C-terminal end of the groove unoccupied.

In contrast to class I MHC molecules, class II MHC molecules bind to longer antigenic peptides. This difference in length can be ascribed to structural differences between the two binding sites and the position of key MHC amino acid residues that interact with the peptide backbone. The few X-ray structures of class II MHC complexes that have been determined so far indicate that the antigenic peptide is stretched out along most of its length and adopts a bound conformation characteristic of a type II polyproline helix. The peptide's N- and C-termini extend out of the binding groove. Side chains of some amino acid residues bind specifically in pockets made of polymorphic MHC amino acid residues while other side chains, spaced

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approximately three amino acid residues apart, point away from the binding site.

Interaction of T cell receptors with both types of MHC complexes involves amino acid residues on both the peptide and MHC molecules. For class I MHC complexes, amino acid residues from the central portion of the peptide, where side chains are most exposed, play a more crucial role in this recognition event, whereas for class II MHC complexes, amino acid residues having side chains pointing up are found along the entire sequence. In designing peptides of this invention, it is preferable to select as anchor amino acid residues those residues with side chains pointing toward T cell receptors.

References for the structures of class I MHC molecules include: Madden, D.R., Garboczi, D.N. & Wiley, D.C. (1993) *Cell* 75, 693-708; Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger, J.L. & Wiley, D.C. (1987) *Nature* 329, 506-512; Madden, D.R., Gorga, J.C., Strominger, J.L. & Wiley, D.C. (1991) *Nature* 353, 321-325; Silver, M.J., Guo, H.-C., Strominger, J.L. & Wiley, D.C. (1992) *Nature* 360, 367-369; Guo, H.-C., Jardetzky, T.S., Garrett, T.P.J., Lane, W.S., Strominger, J.L. & Wiley, D.C. (1992) *Nature* 360, 364-366; and Madden, D.R., Gorga, J.C., Strominger, J.L. & Wiley, D.C. (1992) *Cell* 70, 1035-1048. References for the structures of class II MHC molecules, on the other hand, include Brown, J.H., Jardetzky, T.S., Gorga, J.C., Stern, L.J., Urban, R.G., Strominger, J.L., and Wiley, D.C. (1993) *Nature* 364, 33-39; Stern L.J. and Wiley, D.C. (1994) *Structure* 2, 245-251; and Stern, J.L., Brown, J.H., Jardetzky, T.S., Gorga, J.C., Urban, R.G., Strominger, J.L., and Wiley, D.C. (1994) *Nature* 368, 215-221.

In preparing cyclic peptides of this invention, the cyclization reaction as described in Scheme II below is useful for the synthesis of cyclic peptides on polymer

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supports. Linear chains can also be attached to an antigenic peptide following a strategy analogous to that described in Scheme II where mPEG carboxylic acid is reacted with the deprotected side chains of the lysine residues at anchor positions. The strategy in Scheme II can also be extended to include other symmetrical PEG derivatives and complementary natural and nonnatural amino acid residues on polymer supports for more general applications. For example, the use of PEG diamines and PEG dithiols to link carboxyl and sulfhydryl groups, respectively. In addition, amino acids such as Tyr, Ser, and Thr can react with PEG-epoxide and PEG-isocyanate under basic conditions to form stable ether and urethane linkages, respectively. Similarly, Cys and Orn and Lys can react with PEG-epoxide and PEG-isocyanate to yield stable thioether and urea linkages, respectively.

The peptides of this invention can be used to treat autoimmune diseases such as rheumatoid arthritis (HLA-DR1, -DR4, -DR5 [class II]), multiple sclerosis (HLA-DR2 [class II]); dermatitis herpetiformis (HLA-DR3 [class II]); ankylosing spondylitis (HLA-B27 [class I]); Reiter's diseases (HLA-B27 [class I]); myasthenia gravis (HLA-DR3 [class II]); and insulin dependent diabetes (HLA-DR3, -DR4 [class II]). MHC alleles known to be strongly linked to the above mentioned autoimmune diseases are provided within the parentheses following each of the diseases, and peptides having the correct sequence motif for binding specifically to these alleles can be designed and synthesized in the manners described herein. By the same token, peptides similarly prepared based on the sequence of an antigenic peptide from myelin-associated glycoprotein fragment 556-564 VLFSSIDFRI (class I) or from myelin basic protein fragment 85-99 ENPVVHFFKNIVTPR can be used to treat

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multiple sclerosis. E.g., see Wucherpfennig, K.W. & Strominger (1995), J.L., J. Exp. Med. 181, 1397-1601.

The peptides of this invention can also be used to reduce or prevent graft rejection. Graft rejection is a
5 consequence of T cell-dependent immune responses: T cells from the host recognizes MHC molecules of the graft organ as foreign and proceeds to destroy it. One way to minimize graft rejection is to interfere with the activation of autoreactive T cells by using a peptide
10 designed specifically to be restricted to MHC alleles of the donor.

The dose of a peptide of the present invention for treating an autoimmune disease or minimizing graft rejection varies depending upon the manner of
15 administration, the age and the body weight of the subject and the condition of the subject to be treated, and ultimately will be decided by the attending physician or veterinarian. The peptide may be administered by any route appropriate to the condition being treated. For
20 example, it can be injected into the bloodstream of the subject being treated.

Note that the peptides of the present invention can also be used as a tool to investigate how T cell receptors interact with class I and class II MHC
25 complexes. Results from this type of studies provide guidance to extend the design of peptides of this invention which are more universal in their ability to inhibit T cell.

Without further elaboration, it is believed that
30 one skilled in the art can, based on the description herein, utilize the present invention to its fullest extent. All publications disclosed herein are incorporated by reference. The specific examples set forth below are, therefore, to be construed as merely

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illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

In the following examples, HTLV-1 Tax peptide (LLFGYPVYV) (Utz, U., Koenig, S., Coligan, J.E. & Biddison, W.E. (1992) J. Immunol. 149, 214-221) was modified to produce cyclic MHC-blocking peptides. A reference peptide, a linear Tax peptide control (LLFK[Ac]YPVK[Ac]V), was also prepared, and was tested together with Tax peptide and the cyclic MHC-blocking peptides.

Materials and Methods

Synthesis of PEG derivatives

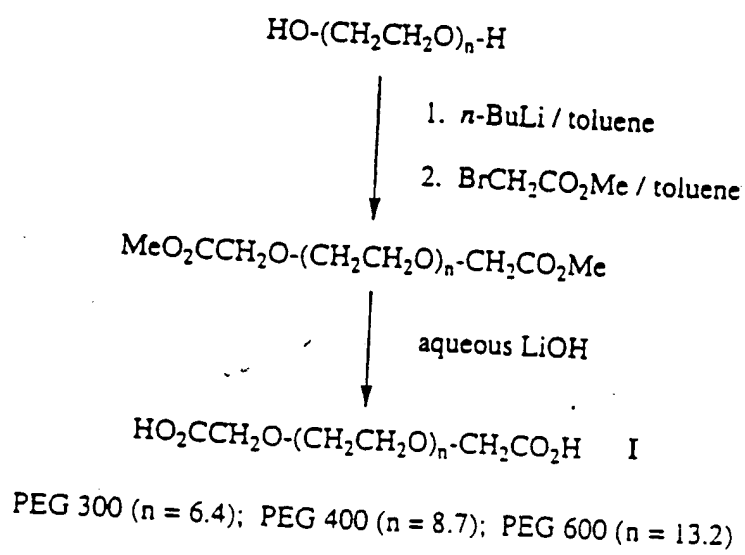
PEG 300 (Union Carbide) (3.99 g, 13.3 mmol) was placed in a oven-dried three-necked flask, equipped with a mechanical stirrer and a dropping funnel, and dissolved in freshly distilled toluene (40 mL) under dry nitrogen (Scheme I). Gehrhardt, H. & Mutter, M. (1987) Polymer Bull. 18, 487-493. Under intermittent cooling in a ice-water bath, a 2.5 M n-butyl lithium solution in hexane (11 mL, 27.5 mmol) (Aldrich Chemical Co.) was slowly injected. Methyl bromoacetate (20.4 mL, 221 mmol) (Aldrich Chemical Co.) was then slowly added through the dropping funnel with rapid stirring. The mixture was stirred overnight at room temperature under nitrogen. The precipitated lithium bromide was filtered and the solvent evaporated to a brownish oil which was then taken up in 30 mL water. The aqueous solution was washed with ether, concentrated in vacuo to half of its volume, and extracted with methylene chloride (DCM). Combined organic extracts were dried over Na₂SO₄ and evaporated to yield 2.7 g oily PEG 300 dimethyl ester (46% yield). Fast atom bombardment (FAB) mass spectrum; [M + H]⁺ 339 (n = 4), 383 (n = 5), 427 (n = 6), 471 (n = 7), 515 (n = 8), 559 (n = 9), 603 (n = 10) where n corresponds to the number of oxyethylene units associated with each of the

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PEG chains in the distribution. Each value of mass-to-charge ratio (m/z) for $[M + H]^+$ ions is shifted by +144 in comparison to the FAB mass spectrum for PEG 300.

- 5 PEG 300 dimethyl ester (2.67 g, 6.0 mmol) was dissolved in 120 mL 1 M aqueous LiOH (120 mmol) and stirred at room temperature for 1.5 hours. The mixture was washed with DCM and the aqueous layer was acidified to pH 1 by the addition of concentrated hydrochloric
10 acid. The aqueous solution was concentrated by evaporation and extracted with DCM. Combined organic extracts were dried over Na_2SO_4 and evaporated to give 1.76 g oily PEG 300 dicarboxylic acid I (70% yield). FAB mass spectrum; $[M + H]^+$ 311 ($n = 4$), 355 ($n = 5$), 399 ($n = 6$), 443 ($n = 7$), 487 ($n = 8$), 531 ($n = 9$), 575 ($n = 10$). Each value of m/z for $[M + H]^+$ ions is shifted by -28 in comparison to FAB mass spectrum for PEG 300 dimethyl ester. PEG 400 and PEG 600 were functionalized as described above and FAB mass spectra showed the
20 expected series of peaks for $[M + H]^+$ ions centered around increasingly higher values of n .

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Scheme I

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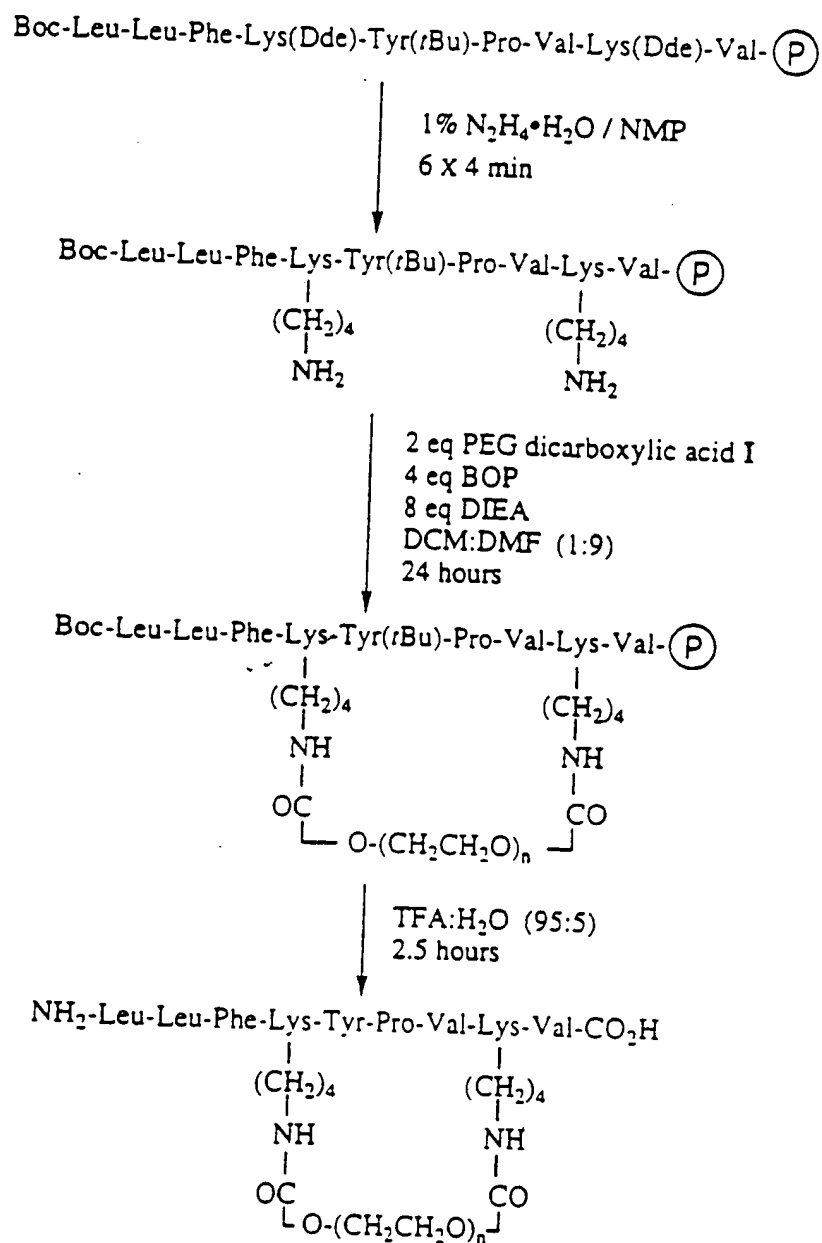
Synthesis and purification of peptides

Cyclic peptides were synthesized manually on 4-hydroxymethylphenoxy (HMP) resin (Applied Biosystems) using N^α-9-fluorenylmethoxycarbonyl (Fmoc) amino acids and N^α-tert-butyloxycarbonyl (tBoc) amino acid for the last residue (Scheme II). Side chains were protected with tert-butyl (tBu) groups except for lysine residues engaged in ring closure which were protected with 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) groups. Bycroft, B.W., Chan, W.C., Chhabra, S.R. & Hone, N.D. (1993) J. Chem. Soc., Chem. Commun. 778-779. Double couplings were performed for each amino acid using preformed hydroxybenzotriazole active esters. Initial substitution level was determined to be 0.25 mmol/g resin by picric acid test. Gisin, B.F. (1972) Anal. Chim. Acta 58, 248-249. After assembly of the protected peptides, selective removal of Dde groups was achieved with 1% hydrazine hydrate in N-methylpyrrolidine (NMP) (6 x 4 min). Deprotection step was shown to be quantitative by picric acid test. Gisin, B.F. (1972) Anal. Chim. Acta 58, 248-249. The peptidyl resin was then divided in three equal parts for cyclization with PEG dicarboxylic acids I. Ring closure was achieved by mixing peptidyl resin suspended in DCM:DMF (1:9) with PEG dicarboxylic I (2 eq) and benzotriazol-1-yloxytris(dimethyl amino)phos-phonium hexafluoro-phosphate (BOP) (4 eq) for 5 min. N,N-diisopropyl-ethylamine (DIEA) (8 eq) was then added and the mixture reacted for 24 hours at room temperature after which a negative ninhydrin test was obtained. Kaiser, E., Colescott, R.L., Bossinger, C.D. & Cook, P.I. (1970) Anal Biochem. 34, 595-598. Cyclization was repeated a second time as above. Similar procedures were used for cyclization with PEG 400 and PEG 600 dicarboxylic acids. Linear Tax and Tax control peptides were synthesized on an automated synthesizer (Applied

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Biosystems 431A) using HMP resin and N^a-Fmoc amino acids. In the synthesis of Tax control, standard side chain protecting groups were used except for lysine residues which were protected with acetyl (Ac) groups.

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Tax 300 ($n = 6.4$); Tax 400 ($n = 8.7$); Tax 600 ($n = 13.2$)

Scheme II

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All peptides were deprotected and cleaved from polymer support by treatment with 95% trifluoroacetic acid (TFA) for 2.5 hours (1.5 hours for linear peptides). Crude peptides were precipitated by the addition of cold ether and were purified by reverse phase high performance liquid chromatography (RP-HPLC) on a Vydac C₁₈ preparative column (2.2 x 25 cm, 15-20 mm) using linear gradients of 0.1% TFA in 90% acetonitrile (ACN) at 10 mL/min. Amino acid analysis: cyclic peptides (average values): Leu 1.91, Phe 1.00, Lys 1.87, Pro 0.92, Tyr very low, Val 1.93; Tax peptide: Leu 1.95, Phe 1.00, Gly 0.98, Pro 0.96, Tyr 1.42, Val 1.86; and Tax control peptide: Leu 2.00, Phe 1.00, Lys 1.98, Pro 0.98, Tyr very low, Val 1.81. FAB mass spectra: Tax 300 peptide: [M + H]⁺ 1336 (n = 3), 1380 (n = 4), 1424 (n = 5), 1468 (n = 6), 1512 (n = 7), 1556 (n = 8), 1600 (n = 9), 1644 (n = 10); Tax peptide: [M + H]⁺ = 1070 (calcd = 1070); and Tax control peptide: [M + H]⁺ = 1190 (calcd = 1190). The FAB mass spectra for Tax 400 and Tax 600 peptides showed expected series of peaks for [M + H]⁺ ions centered around increasingly higher values of n.

Enzymatic hydrolysis of cyclic peptides

Tax 300 peptide (1 mM) was incubated at 30°C with proline specific endopeptidase (Seikagaku Co.) at enzyme:substrate ratio of 1: 900 (w/w) in 25 mM phosphate buffer (pH = 7.0). Aliquots from digest were taken at different times and analyzed by RP-HPLC on a Vydac C₄ column (0.46 x 25 cm, 5 mm) using a linear gradient of 0.1% TFA in 90% ACN at 1.0 mL/min. Fractions corresponding to the digested peptide were collected, evaporated, and lyophilized. FAB mass spectrum; [M + H]⁺ 1398 (n = 4), 1442 (n = 5), 1486 (n = 6), 1530 (n = 7), 1574 (n = 8), 1619 (n = 9).

In vitro assembly and purification of class I MHC

complexes Folding and assembly of class I MHC complexes

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was initiated by dilution of denatured E. coli-expressed human heavy chain (HLA-A*0201) (2 mM) and b₂-microglobulin (b₂m) (1 mM) in the presence of excess synthetic peptides (10-30 mM) and the mixture incubated at 10°C for approximately 48 hours. Garboczi, D.N., Hung, D.T. & Wiley, D.C. (1992) Proc. Natl. Acad. Sci. USA 89, 3429-3433. The crude concentrate was purified by gel filtration HPLC on a Phenomenex Biosep Sec-3000 column using 20 mM Tris-HCl (pH = 7.5) containing 150 mM NaCl at 0.8 mL/min. Fractions corresponding to class I MHC complexes were collected, concentrated, and diluted with 10 mM MOPS (pH 7.5). Concentrations of stock solutions were determined by amino acid analysis, using norleucine as internal standard, and spectrophotometrically using a value of 94,240 M⁻¹cm⁻¹ at 280 nm.

Thermal denaturation of class I MHC complexes

Circular dichroism (CD) studies were done using a 1 mm cell on an Aviv 62DS spectropolarimeter equipped with a thermoelectric temperature controller. Thermal denaturation curves for HLA-A2 complexes (0.18 mg/mL) were obtained in triplicate by monitoring the change in CD signal at 218 nm in the range 20°C-90°C using a scan rate of 0.7 °C/min. Bouvier, M. & Wiley, D.C. (1994) Science 265, 398-402. Averaged denaturation curves were fit by a nonlinear least squares analysis to determine melting temperatures. Bouvier, M. & Wiley, D.C. (1994) Science 265, 398-402.

Results

Peptide design and synthesis

The key element in the design of cyclic peptides is the presence of large and floppy nonpeptidic loops favored to project out of the binding groove in such a way that the antigenic region of class I MHC complexes becomes inaccessible for recognition by T cell receptors. Design was primarily guided from knowledge of the

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conformation of several nonamers, including the Tax peptide, bound in the groove of HLA-A2 as revealed by X-ray crystallography. Madden, D.R., Garboczi, D.N. & Wiley, D.C. (1993) Cell 75, 693-708. Peptide side chains at positions 4 and 8 are generally shown pointing up and therefore ideal anchor sites for the substitution of lysine residues. High molecular weight PEG dicarboxylic acids were used to covalently link the side chains of lysine residues resulting in large ring structures that include all residues predicted to contact the T cell receptor. The excellent water solubility of the repeating oxyethylene group favors extension of PEG loops out of the binding site and towards T cell receptors. The peptide amino and carboxyl termini and peptide anchor residues at position 2 (Leu) and position 9 (Val) have been unmodified to favor high-affinity binding and to retain specificity of interactions, respectively, between cyclic peptides and class I MHC molecules. Bouvier, M. & Wiley, D.C. (1994) Science 265, 398-402.

Functionalization of commercially available PEGs into useful derivatives (Scheme I) was achieved essentially as reported for monomethoxypoly(ethylene glycol) (mPEG). Gehrhardt, H. & Mutter, M. (1987) Polymer Bull. 18, 487-493. In brief, the lithium salt of hydroxyl end groups was alkylated with methyl bromoacetate followed by alkaline hydrolysis to yield desired symmetrical PEG dicarboxylic acids I. The FAB mass spectra for intermediates and products were consistent with the structures. Starting materials of different average MWs were used; PEG 300, PEG 400, and PEG 600, which correspond to an average number of repeating oxyethylene unit (n value) of 6.4, 8.7, and 13.2, respectively. It should be noted that all PEGs contain a narrow distribution of chains of different MWs.

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Tax peptide was synthesized on HMP resin (Scheme II) using a strategy that includes N^α-Fmoc amino acids, N^α-Boc amino acid for the last residue, and Dde protecting groups for the side chains of lysine residues engaged in ring closure. Selective cleavage of Dde groups was achieved quantitatively with 1% hydrazine hydrate in NMP followed by cyclization of the liberated lysine side chains via PEG dicarboxylic acids I. In this novel cyclization strategy, ring formation occurs on the polymer support in a single step using a symmetrical homobifunctional spacer. The symmetrical nature of PEG molecules ensures that structurally homogeneous cyclic peptides are obtained. In all studies, the cyclization reaction proceeded in DCM:DMF (1:9) using BOP activation method and was complete after 24 hours as indicated by a negative ninhydrin test. Kaiser, E., Colescott, R.L., Bossinger, C.D. & Cook, P.I. (1970) Anal Biochem. 34, 595-598. Final deprotection and cleavage of the peptide from the polymer support was done by treatment with 95% TFA to afford desired cyclic peptides (Tax 300, Tax 400, and Tax 600) with ring size varying between about 44 atoms (Tax 300) and 65 atoms (Tax 600).

Peptide characterization

Crude cyclic peptides were purified by RP-HPLC using a C₁₈ preparative column and eluted either as sharp (Tax 300 and Tax 400) or rather broad (Tax 600) peaks. In all cyclization reactions studied the desired cyclic monomeric peptides were obtained as the major product with no evidence for formation of cyclodimers as analyzed by RP-HPLC. Formation of cyclodimers and cyclooligomers during cyclization of peptides on polymer support depends on a mixed combination of factors such as the type of polymer support, the peptide substitution level, and the nature and kinetics of the cyclization reaction. Rothe, M., Sander, Fischer, W. Mastle, W. & Nelson, B.

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(1978) in Peptides, Proc. 5th Amer. Pept. Symp., eds. Goodman, M. & Meienhofer, J. (Pierce Chem. Co., Rockford, Il), pp. 506-509; and Bouvier, M. & Taylor, J.W. (1992) in Peptides, Proc. 12th Amer. Pept. Symp., eds. Smith, J.A. & Rivier, J.E. (Escom Science Publishers B.V., Leiden, The Netherlands), pp. 535-536. Sequence composition was confirmed by amino acid analysis and the presence of PEG was demonstrated by FAB mass spectrometry. Fig. 1 shows a typical FAB mass spectrum for purified Tax 300 peptide revealing a series of distinct peaks corresponding to m/z for $[M + H]^+$ peptide ions. Each peak is separated by intervals of m/z 44, corresponding to the mass of the repeating oxyethylene group, and is identified by the appropriate n value. The distribution of peaks in Fig. 1 is centered between $n = 6$ and $n = 7$ as obtained in the FAB mass spectrum for PEG 300 dicarboxylic acid I (spectrum not shown) suggesting that the cyclization reaction is compatible with all PEG chains in the distribution. Similar results were obtained for PEG 400 and PEG 600 dicarboxylic acids I.

The cyclic nature of peptides was further confirmed from digestion of Tax 300 peptide by proline specific endopeptidase. This enzyme cleaves specifically peptide bonds on the carboxyl side of proline residues. Yoshimoto, T., Walters, R. & Tsuru, D. (1980) J. Biol. Chem. 255, 4786-4792. Hydrolysis of the Pro-Val bond as found in the ring structure of cyclic peptides (Scheme II, bottom) should produce a single linear peptide characterized by the additional weight of a water molecule. RP-HPLC of two aliquots from proline specific endopeptidase digestion of Tax 300 peptide taken at different times was conducted. The digest after 3.5 hours reveals the presence of an additional single peak, which became relatively more dominant after 16.5 hours. Analysis of the purified digested peptide by FAB mass

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spectrometry shows a series of peaks for $[M + H]^+$ ions shifted by m/z 18 in comparison to the FAB mass spectrum for the undigested peptide (Fig. 1). These results support the structure of cyclic peptides as shown in Scheme II, bottom.

In vitro assembly of class I MHC complexes

HLA-A2 complexes were reconstituted from human heavy chain and b_2m expressed in *E. coli* in the presence of excess cyclic peptides and purified by gel filtration chromatography (Fig. 2). Garboczi, D.N., Hung, D.T. & Wiley, D.C. (1992) Proc. Natl. Acad. Sci. USA 89, 3429-3433. All cyclic peptides promoted folding of class I MHC complexes as indicated by the presence of a single peak, identified by an arrow, at the expected elution time (13 min). Adjacent peaks are aggregated heavy chain (7.5 min) and free b_2m (16.5 min). Analysis of purified HLA-A2 complexed with Tax 300 peptide by FAB mass spectrometry (Fig. 3) shows the expected series of peaks for $[M + H]^+$ peptide ions (compare Fig. 1 and Fig. 3) confirming the association of cyclic peptides with HLA-A2. In addition, results show that the distribution of peaks is identical in both Fig. 1 and Fig. 3 suggesting that folding and assembly of HLA-A2 occurs independently of the particular size of the peptide ring structure. A series of unidentified peaks corresponding to $[M + Na]^+$ peptide ions is also clearly apparent in Fig. 3. The relatively high intensity background in Fig. 3 is typical for low concentration FAB mass spectrometry analysis of class I MHC complexes. Similar results were obtained for HLA-A2 complexed with Tax 400 and Tax 600 peptides.

Thermal denaturation experiments were done to determine the effect of the peptide ring structure on the stability of HLA-A2 complexes. To this end, Tax peptide (LLFGYPVYV) and Tax control peptide (LLFK(Ac)YPVK(Ac)V)

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were synthesized and used in reconstitution experiments to assemble HLA-A2. Denaturation curves for complexes were obtained by monitoring the change in CD signal at 218 nm and values for the midpoint of the unfolding transition, melting temperature or T_m , generated from a nonlinear least squares analysis of thermal denaturation curves (Bouvier, M. & Wiley, D.C. (1994) Science 265, 398-402) are listed in Table 1. The results show that melting temperatures for HLA-A2 complexed with cyclic peptides are similar and range between 70.7°C and 72.1°C suggesting that stabilization of complexes is independent on the particular size of the peptide ring structure. Results from Table 1 also indicate that the stability of these complexes is comparable to that of HLA-A2 complexed with the Tax control peptide ($T_m = 69.8^\circ\text{C}$). Since measurement of melting temperatures by CD has been shown to correlate with peptide binding affinity for class I MHC complexes (Fahnestock, M.L., Tamir, I., Nahri, L. & Bjorkman, P.J. (1992) Science 258, 1658-1662; and Bouvier, M. & Wiley, D.C. (1994) Science 265, 398-402), these results suggest that cyclic peptides and Tax control peptide adopt similar bound conformations with most likely the same contacts made to MHC residues. The presence of PEG loops thus appear to have no destabilizing effect on the structure of class I MHC complexes. Crystals of HLA-A2 complexed with cyclic peptides have been obtained and determination of X-ray structures could reveal whether the aqueous environment solubilizes PEG loops and favors a most stable conformation.

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Table 1

	Peptide	T _m (± 1.0°C)
5	Tax 300	70.7
	Tax 400	71.6
	Tax 600	72.1
	Tax control	69.8
	Tax	73.6
10		

OTHER EMBODIMENTS

From the above description, one skilled in the art can easily ascertain the essential characteristics of the present invention, and without departing from the spirit
15 and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. For examples, the anchor amino acid residue of an antigenic nonapeptide of a T cell receptor on CD⁸⁺ T cells can be at position 1, rather than
20 position 4, 5, 6, 7, or 8. Also contemplated within the scope of this invention are peptide analogs obtained by back modification of the above-described MHC-blocking peptides, e.g., replacement of at least one of the peptide bonds with -CH₂-NH-, CH₂-S-, or the like. Thus,
25 other embodiments are also within the claims.

What is claimed is:

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CLAIMS

1. A synthetic peptide that blocks the interaction of a T cell receptor on T cells with an MHC molecule, said peptide containing 8, 9 or 10 amino acid residues
5 and being identical to an antigenic peptide of said T cell receptor except that a linker is covalently bonded to a first amino acid residue and a second amino acid residue of said peptide to form a 20- to 200-membered ring, and that each of said first and second amino acid
10 residues contains a side chain with a functional group selected independently from the group consisting of an amino group, a carboxyl group, a hydroxyl group, and a sulfhydryl group, wherein said first and second amino acid residues are covalently bonded to said linker via
15 said functional groups; provided that: if said peptide contains 8 amino acid residues, each of said first and second amino acid residues is at one of positions 3-8; if said peptide contains 9 amino acid residues, each of said first and second amino acid residues is at one of
20 positions 4-8; and if said peptide contains 10 amino acid residues, each of said first and second amino acid residues is at one of positions 4-9.

2. The peptide of claim 1, wherein said peptide contains 8 amino acid residues, and each of said first
25 and second amino acid residues is at one of positions 4-8.

3. The peptide of claim 2, wherein said first and second amino acid residues are apart by 2 or 3 amino acid residues.

30 4. The peptide of claim 3, wherein said linker is covalently bonded to said first and second amino acid residues to form a 40- to 120-membered ring.

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5. The peptide of claim 4, wherein said linker is a bifunctional poly(ethylene glycol).

6. The peptide of claim 5, wherein each of said first and second amino acid residues is selected independently from the group consisting of Lys, Orn, Glu, Asp, Ser, Thr, Tyr, and Cys.

7. The peptide of claim 1, wherein said peptide contains 8 amino acid residues, and said linker is covalently bonded to said first and second amino acid residues to form a 40- to 120-membered ring.

8. The peptide of claim 7, wherein said linker is a bifunctional poly(ethylene glycol).

9. The peptide of claim 8, wherein each of said first and second amino acid residues is selected independently from the group consisting of Lys, Orn, Glu, Asp, Ser, Thr, Tyr, and Cys.

10. The peptide of claim 1, wherein said peptide contains 9 amino acid residues, and said first and second amino acid residues are apart by 2 or 3 amino acid residues.

11. The peptide of claim 10, wherein said linker is covalently bonded to said first and second amino acid residues to form a 40- to 120-membered ring.

12. The peptide of claim 11, wherein said linker is a bifunctional poly(ethylene glycol).

13. The peptide of claim 12, wherein each of said first and second amino acid residues is selected

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independently from the group consisting of Lys, Orn, Glu, Asp, Ser. Thr, Tyr, and Cys.

14. The peptide of claim 1, wherein said peptide contains 9 amino acid residues, and said linker is
5 covalently bonded to said first and second amino acid residues to form a 40- to 120-membered ring.

15. The peptide of claim 14, wherein said linker is a bifunctional poly(ethylene glycol).

16. The peptide of claim 15, wherein each of said
10 first and second amino acid residues is selected independently from the group consisting of Lys, Orn, Glu, Asp, Ser. Thr, Tyr, and Cys.

17. The peptide of claim 1, wherein said peptide contains 10 amino acid residues, and each of said first
15 and second amino acid residues is at one of positions 5-8.

18. The peptide of claim 17, wherein said first and second amino acid residues are apart by 2 amino acid residues.

20 19. The peptide of claim 18, wherein said linker is covalently bonded to said first and second amino acid residues to form a 40- to 120-membered ring.

20. The peptide of claim 19, wherein said linker is a bifunctional Poly(ethylene glycol).

25 21. The peptide of claim 20, wherein each of said first and second amino acid residues is selected

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independently from the group consisting of Lys, Orn, Glu, Asp, Ser, Thr, Tyr, and Cys.

22. The peptide of claim 1, wherein said peptide contains 10 amino acid residues, and said linker is
5 covalently bonded to said first and second amino acid residues to form a 40- to 120-membered ring.

23. The peptide of claim 22, wherein said linker is a bifunctional poly (ethylene glycol).

24. The peptide of claim 23, wherein each of said
10 first and second amino acid residues is selected independently from the group consisting of Lys, Orn, Glu, Asp, Ser, Thr, Tyr, and Cys.

25. A synthetic peptide that blocks the interaction of a T cell receptor on T cells with an MHC
15 molecule, said peptide containing 8, 9 or 10 amino acid residues and being identical to an antigenic peptide of said T cell receptor except that one, two, or three chains, each 10-200 atoms in length, are respectively
20 linked via covalent bonding to one, two, or three amino acid residues of said peptide, and that each of said one, two, or three amino acid residues has a side chain with a functional group selected independently from an amino group, a carboxyl group, a hydroxyl group, and a
25 sulfhydryl group, wherein said one, two, or three chains are covalently linked to said functional groups of said one, two, or three amino acid residues; provided that: if said peptide contains 8 amino acid residues, each of said one, two, or three amino acid residues is at one of positions 3-8; if said peptide contains 9 amino acid
30 residues, each of said one, two, or three amino acid residues is at one of positions 4-8; and if said peptide

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contains 10 amino acid residues, each of said one, two, or three amino acid residues is at one of positions 4-9.

26. The peptide of claim 25, wherein said peptide contains 8 amino acid residues, two or three chains are
5 respectively linked via covalent bonding to two or three amino acid residues of said peptide, and each of said first and second amino acid residues is at one of positions 4-8.

27. The peptide of claim 26, wherein each of said
10 two or three chains is 20-100 atoms in length.

28. The peptide of claim 27, wherein said linker is a bifunctional poly(ethylene glycol).

29. The peptide of claim 28, wherein each of said two or three amino acid residues is selected
15 independently from the group consisting of Lys, Orn, Glu, Asp, Ser, Thr, Tyr, and Cys.

30. The peptide of claim 25, wherein said peptide contains 9 amino acid residues, and two or three chains are respectively linked via covalent bonding to two or
20 three amino acid residues of said peptide.

31. The peptide of claim 30, wherein each of said two or three chains is 20-100 atoms in length.

32. The peptide of claim 31, wherein said linker is a bifunctional poly(ethylene glycol).

25 33. The peptide of claim 32, wherein each of said two or three amino acid residues is selected

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independently from the group consisting of Lys, Orn, Glu, Asp, Ser, Thr, Tyr, and Cys.

34. The peptide of claim 25, wherein said peptide contains 10 amino acid residues, two or three chains are
5 respectively linked via covalent bonding to two or three amino acid residues of said peptide, and each of said first and second amino acid residues is at one of positions 5-8.

35. The peptide of claim 34, wherein each of said
10 two or three chains is 20-100 atoms in length.

36. The peptide of claim 35, wherein said linker is a bifunctional poly(ethylene glycol).

37. The peptide of claim 36, wherein each of said two or three amino acid residues is selected
15 independently from the group consisting of Lys, Orn, Glu, Asp, Ser, Thr, Tyr, and Cys.

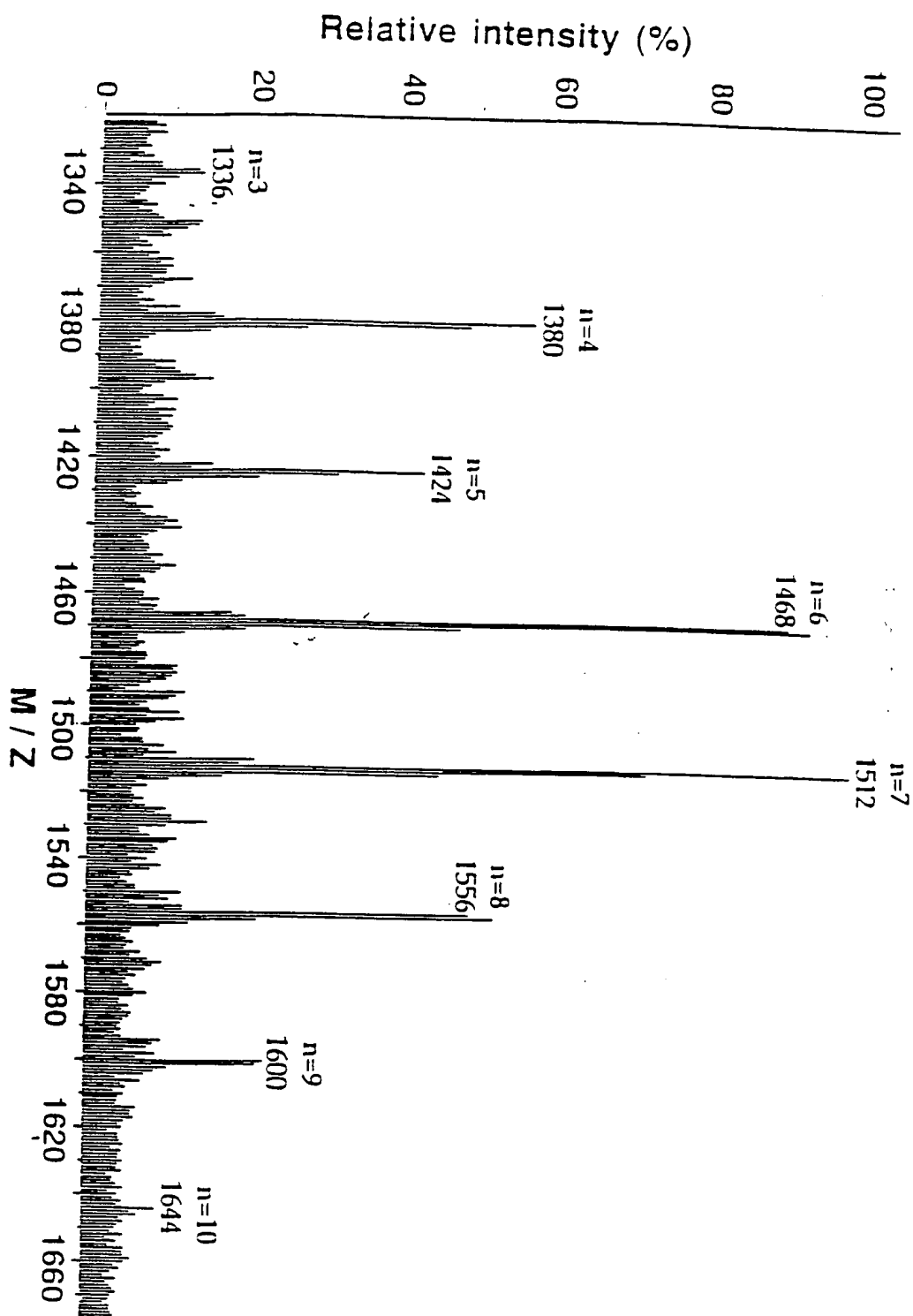


FIGURE 1

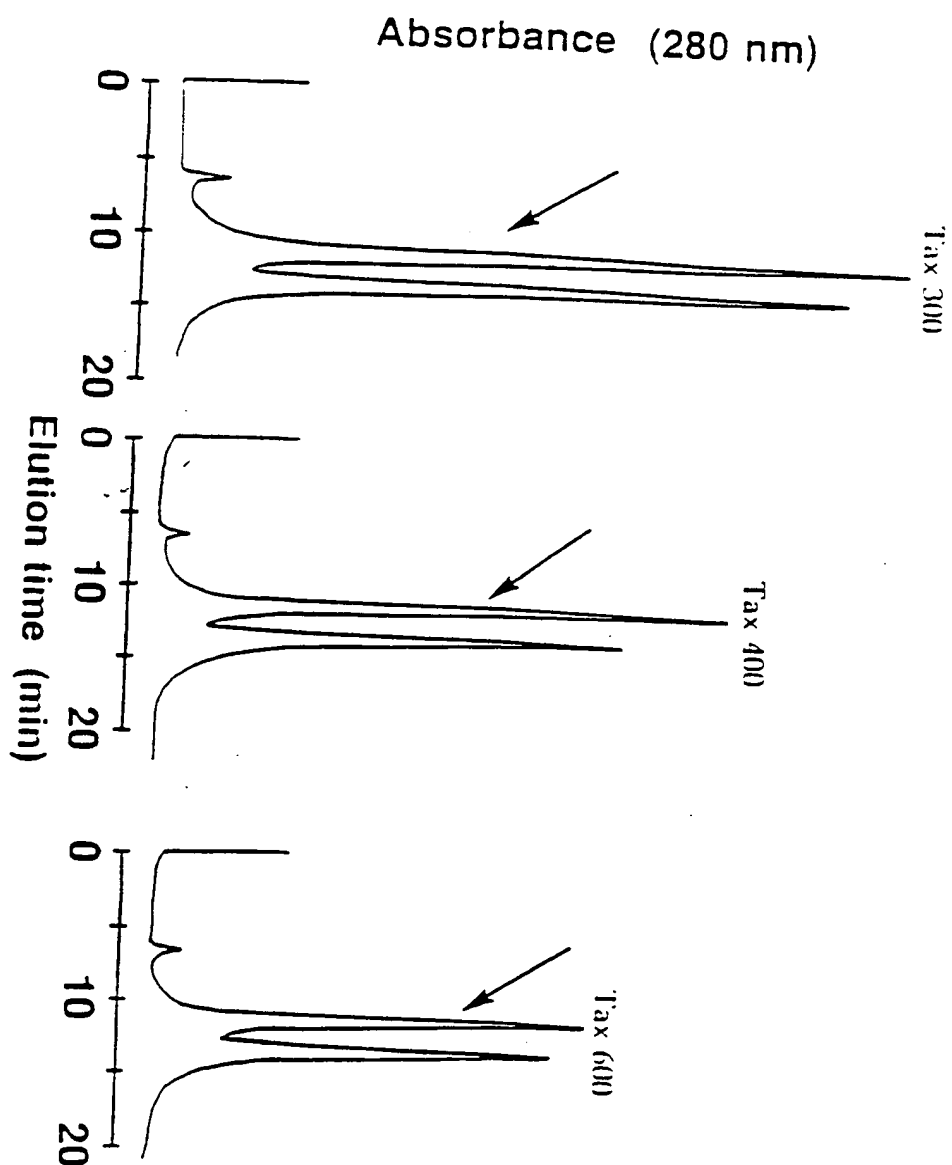


Fig. 1. Folding and assembly of IILA-A2 complexes. Gel filtration chromatograms of IILA-A2 complexed with Tax 300, Tax 400, and Tax 600 peptides. Each complex eluted as a single peak identified by an arrow.

FIGURE 2

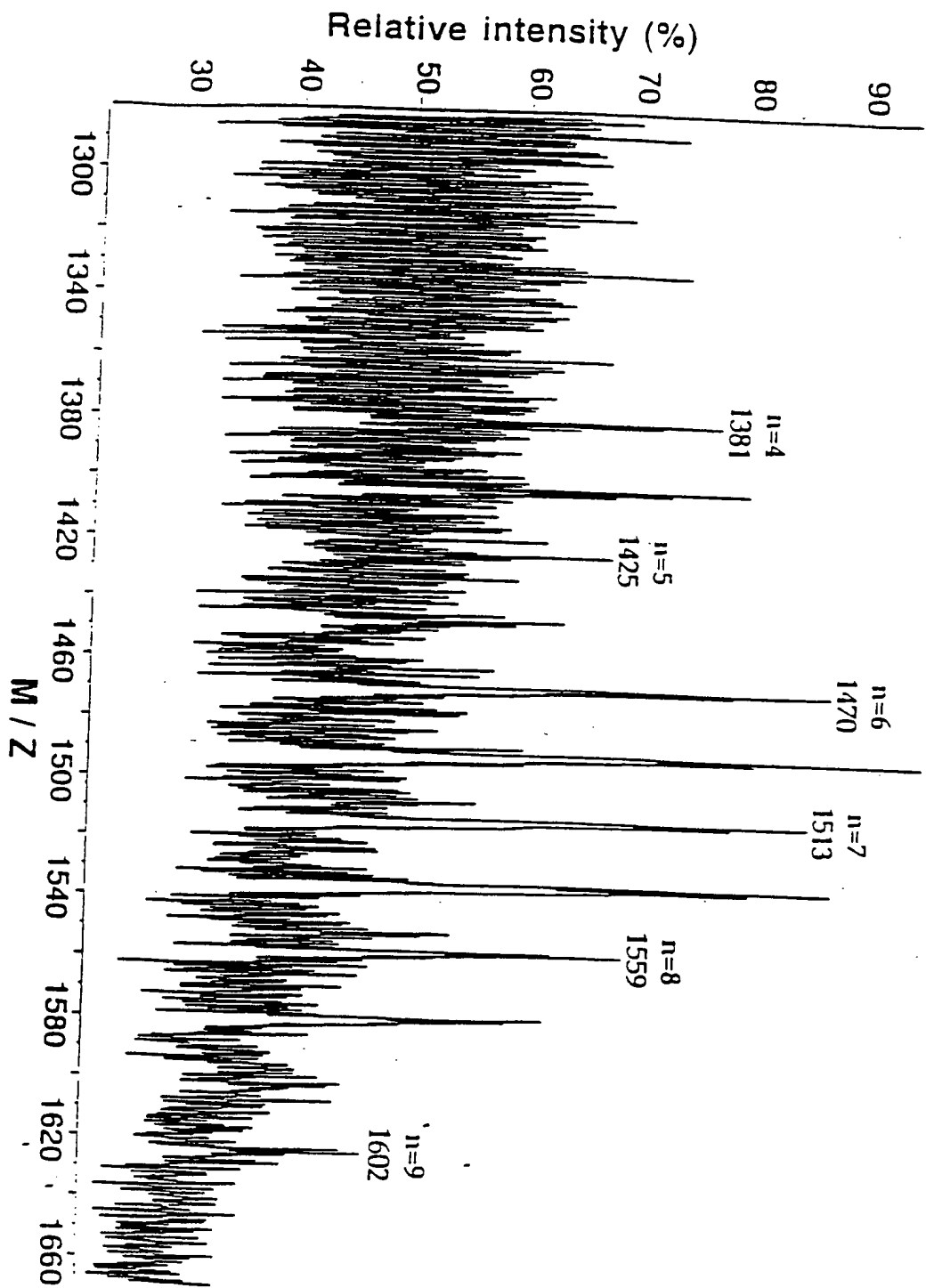


FIGURE 3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/10396

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : 424/184.1, 194.1; 514/15, 16; 530/317, 323

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 194.1; 514/15, 16; 530/317, 323

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	BOUVIER et al. Antigenic peptides containing large PEG loops designed to extend out of the HLA-2 binding site form stable complexes with class I major histocompatibility complex molecules. Proceedings of National Academy of Science U.S.A. May 1996, Vol. 93, No. 10, pages 4583-4588. entire document	1-37
Y	ROGNAN et al. Rational design of nonnatural peptides as high-affinity ligands for HLA-B*2705 human leukocyte antigen. Proceedings of National Academy of Sciences U.S.A. January 1995, Vol. 92, pages 753-757. entire document	1-37

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 AUGUST 1996	Date of mailing of the international search report 27 SEP 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MARTHA T. LUBET Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/10396

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FOX et al. Functionally distinct agretopic and epitopic sites. Analysis of the dominant T cell determinant of moth and pigeon cytochromes c with the use of synthetic peptide antigens. Journal of Immunology. 01 September 1987, Vol. 139, pages 1578-1588. entire document	1-37
Y	SEHON Suppression of antibody responses by conjugates of antigens and monomethoxypoly(ethylene glycol). Advanced Drug Delivery Reviews. 1991, Vol. 6, pages 203-217. entire document	1-37
Y	ABUCHOWSKI et al. Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. Journal of Biological Chemistry. 10 June 1977, Vol. 252, pages 3578-3581. entire document	1-37
Y	ZALIPSKY. Functionalized poly (ethylene glycol) for preparation of biologically relevant conjugates. Bioconjugate Chemistry. 1995, Vol. 6, No. 2, pages 150-165. entire document	1-37
Y	JORGENSEN et al. Molecular components of T-Cell recognition. Annual Review of Immunology. 1992, Vol. 10, pages 835-873. entire document	1-37

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/10396

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A61K 39/00, 39/38, 39/385, 38/12; C07K 5/00, 7/00, 16/00, 17/00

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG, MEDLINE, EMBASE, BIOSIS DERWENT WORLD PATENT
T CELL RECEPTOR, HISTOCMPATIBLITY COMPLEX, BLOCKS (5N)INTERACTION,
INHIBIT(5N)INTERACTION, POLY (ETHYLENE GLYCOL), PEPTIDE BINDING